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Late cellular effects of ^{12}C ions

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Summary. — The expanding role of particle-based radiation treatment of cancer (hadrontherapy) requires a thorough understanding of its possible late effects at the cellular and tissue levels. The biological effectiveness of a ^{12}C ion beam in the induction of two biological endpoints, neoplastic transformation and premature cellular senescence was studied in human cells *in vitro* and compared to that exhibited by low linear energy transfer (LET) radiation. Cells were exposed to the therapeutic ^{12}C ion beam used at GSI at various positions of the beam ionisation path, corresponding to the low-LET entrance channel (plateau), the high-LET Spread-Out Bragg Peak (SOBP) or at 3.8 cm past the SOBP. In the framework of the INFN-funded project ETIOPE, this paper reports preliminary results on survival and neoplastic transformation after a physical dose of 0.75 Gy, and on the onset of cellular senescence after 0.5 or 2 Gy of plateau- and SOBP-irradiated cells. A three-dimensional approach to investigate the effects ^{12}C ion beams on the expression of cell adhesion molecules is also examined.

PACS 87.53.-j – Effects of ionizing radiation on biological systems.
PACS 87.53.Jw – Therapeutic applications, including brachytherapy.
PACS 87.19.xj – Cancer.
PACS 87.55.dh – Tissue response.

1. – Introduction

The use of protons and heavier charged particles such as ^{12}C ion beams in the treatment of cancer, commonly referred to as hadrontherapy [1], may entail significant therapeutic gains compared to conventional radiotherapy (photons) because of the physical

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properties of dose deposition within the irradiated tissue as described by Bragg's curve, and the ion beams' radiobiological properties [2]. The former allows a greater sparing of the healthy tissue as the dose deposited rises steeply as the particles' range nears its end reaching a maximum (Bragg peak), which can be modulated in order to encompass the tumour linear dimensions. Furthermore, a reasonably homogenous biologically effective dose can be achieved along such a Spread-Out Bragg Peak (SOBP), which, in the case of ^{12}C ions, elicits a higher Relative Biological Effectiveness (RBE) compared to photons as well as protons in the induction of curative endpoints such as tumour cell inactivation [3]. Carbon ion-based hadrontherapy therefore represents a useful approach to eradicating tumours that are otherwise resilient to photon therapy. However, the existing paucity of data on the RBE for adverse long-term effects due to ion beam exposure, such as cancer or tissue degeneration [4], represents a source of uncertainty in light of a wider use of hadrontherapy. Knowledge of late effects of ion beam irradiation is needed for both the Linear Energy Transfer (LET) values prior to the SOBP as well as those immediately beyond it. The healthy tissue exposed in the entrance beam channel will inevitably receive a dose, which albeit reduced compared to that due to a photon beam and 2-3 fold less than that released in the SOBP, is likely to be biologically relevant because of the great effectiveness of the ions at causing serious lesions (damage clusters) to the cell's DNA. Likewise, despite the rapid decrease in the energy released after the SOBP, the fragmentation of the incident beam with the nuclei of the biological targets may result in the normal cells of the tissue seated beyond the end of the beam penetration range being hit by slowed-down fragments [5], hence having higher linear energy transfer (LET) than the pristine beam and of unknown biological relevance.

Cancer is a well-known somatic late effect of ionising radiation. Photon radiotherapy has been shown to be associated with a significant enhancement in the risk of secondary tumour induction in long-term survivors [6]. With heavy ions, no experience is available to estimate risk of second malignancies induction, an aspect of great concern in the case of the treatment of younger patients because of their longer life span. The development of *in vitro* oncogenic transformation assay systems has made it possible to assess, at the cellular level, the carcinogenic potential of a variety of physical and chemical agents. These systems are particularly suitable for comparing different agents or treatment modalities risk [7]. Additional advantages are that cell survival can be determined in parallel to transformation, the cells can be irradiated in track-segment conditions and they can be used to investigate definite positions within a certain radiation field. The human hybrid (Hela X skin fibroblast) cell line, developed by Stanbridge *et al.* [8] and Redpath *et al.* [9] and designated CGL1 has been used to compare carbon ion beams to conventional photon irradiation. In a first phase monoenergetic ^{12}C ion beams of energies between 270 and 11.4 MeV/n (LET values between 13.8 and 172 keV/μm) were investigated [10]; more recently, the irradiation of an extended tumour volume has been simulated and survival, neoplastic transformation and micronuclei induction have been measured in different positions of the ionisation profile along the beam penetration direction [11, 12].

As opposed to the extensively studied cancer-related long-term effects, non-cancer degenerative diseases induced by ionising radiation have been only recently attracting attention as a source of health concern. It is known that high-energy heavy ions are particularly efficient at causing degenerative alterations, showing a high RBE for cataract and neurodegenerative diseases [13], hence possessing a capacity of accelerating the physiological ageing of the exposed tissues. More recently, analysis of data from Hiroshima and Nagasaki survivors has confirmed a significant increase in the incidence of cardiovascular mortality [14]. Although these data refer to doses higher than those hadrontherapy

patients will incur, it is nevertheless necessary to reduce the uncertainty surrounding the RBE of charged particles for such late effects thereby optimising treatment plans.

Ionising radiation has been reported to promote a state of apparent cell-cycle arrest in both normal and tumour cell lines closely resembling the senescent phenotype [15-17]. The latter is the physiological fate incurred by all somatic primary cell lines when cultured *in vitro* because of the exhaustion of their proliferative potential but there is convincing evidence that such a phenotype occurs also *in vivo* and is related to the accumulation of cells containing shortened telomere sequences [18]. However, many stimuli, at sub-lethal doses, can ectopically provoke the senescence pathway [19]. Although DNA damage is thought to be the main effector of the senescent-like fate [15], the underlying mechanisms of stress-induced premature senescence (SIPS) are not understood [20, 21]. It may be triggered by oncogene(s) [22]: premature entry into senescence would thus represent a stress-induced avoidance of transformation; it could be associated with telomere shortening [23], in which case, since reduction of telomere length below a critical threshold is thought to result in telomere-telomere fusion, widespread genome instability and increased tumourigenic risk would ensue. Moreover, the role of SIPS in radiotherapy needs to be ascertained [21].

Since the ubiquitous endothelial tissue represents one of the chief targets of tissue-related effects of irradiation as demonstrated by the capillary-initiated radiopathogenesis of several organs [24], the ectopic onset of senescence and its relationship with telomere length was studied in human umbilical vein endothelial cells (HUVECs). HUVECs have been widely used for investigating angiogenesis and tumourigenesis of the endothelium [25]. They are known to show progressive telomere shortening with time in culture [26]. Previously, ^{12}C ion-induced clonogenic cell death was determined in this system [27]. The therapeutic beam was shown to be 2-fold and 3-fold more effective at cell killing than X-rays in the plateau region and at the SOBP, respectively. In this study, senescence was revealed by positivity for β-galactosidase activity according to the biomarker assay established by Dimri *et al.* [28] while cytogenetic analysis of telomeres was performed by Interphase Quantitative (IQ)- FISH, adapting a methodology originally developed by others [29,30]. By both assays, the effectiveness of ^{12}C ions at causing HUVECs to senesce prematurely was studied and compared to that exhibited following X-ray irradiation.

Most of experimental data on the effects of ionising radiation have relied on conventional *in vitro* cell culture techniques based on cell monolayers. The need of furthering radiobiological studies to three-dimensional systems that better model the complex cell-to-cell signalling and interplay deriving from the *in vivo* tissue architecture is widely acknowledged, an example of which is provided by the spheroid system [31]. The set-up of a spheroid-based approach to compare previous results on gamma-ray–induced modifications of cell adhesion molecules of interest in endothelial tissue late effects with those from ^{12}C ion irradiation will be discussed.

2. – Experimental procedure

2˙1. *Neoplastic transformation.* – CGL1 cells are not tumourigenic when injected in nude mice whereas transformed cells form carcinomas. Expression of tumourigenicity in these cells is associated with the expression of a cell surface protein, which has been identified as an intestinal alkaline phosphatase IAP [32]. This protein is readily detected by the alkaline phosphatase chromogenic substrate, Western Blue. Therefore, blue foci of transformed cells are scored against the white background of the normal cells. Details on

cell culture, transformation assay protocol and irradiation procedure have been reported previously [10, 11, 33]. Briefly, about 20 h before irradiation, exponentially growing cells were seeded into standard tissue culture (T25) flasks. Immediately after irradiation, cells were removed by trypsinization and then plated in T75 flasks at a cell concentration such that the resulting number of viable cells was as close to 50 viable cells per cm^2 as possible. After 21 days of incubation, with the growth medium changed once a week, the cultures were fixed and stained with Western Blue. For each experiment in parallel with the transformation assay small aliquots of the cell suspensions were also plated in T25 flasks for the evaluation of the surviving fraction allowing colonies to grow for about 12 days after irradiation.

2˙2. *Cellular senescence.* – Early passage HUVECs were irradiated at the SIS facility in Darmstadt using a 270 MeV/n carbon ion beam representing the plateau (LET $\approx$ 13 keV/μm) region and at the centre of a 1 cm SOBP (LET $\approx$ 100 keV/μm) at a depth of approximately 35 mm as to examine the senescence-related late effects in both the healthy tissue at the beam entrance channel and in the tumour-associated endothelium. Two doses were chosen for each position, one of relatively sub-lethal magnitude (0.5 Gy) and the other known to reduce HUVEC survival by one log compared to the former for either LET value [27], *i.e.* 2 Gy. X-rays were used as a reference, doses being chosen as to give approximately the same clonogenic survival as previously determined [27]. At the first passage post-irradiation, irradiated flasks were sub-cultured taking into account estimated clonogenic survival. Each sample was thus split, serially cultivated and, at regular intervals, either harvested or replated. Harvested samples were either processed for β-galactosidase assay as directed by manufacturer (Sigma-Aldrich, USA) or for IQ-FISH. The latter was carried out by analysing interphase cells on slides that were co-hybridised with a pantelomeric probe (Dako, Denmark) and a centromeric probe (Olympus, Italy), specific for chromosome 12. The assumption is that the intensity of the fluorescence emitted by telomere-conjugated probes is proportional to telomere length [30]. The two probes emit in the red and green portion of the visible spectrum respectively when examined at the fluorescence microscope. In-house fluorescence signal analysis was used by opportunely adapting a classifier for automated metaphase search (software by MetaSystems, Germany): single cells were localised and signals were acquired by scanning ten planes around the mid-focal plane at 1.5 μm steps. The ratio of the red fluorescence intensity (telomeres) was divided by the green fluorescence intensity (centromere) as to normalise the telomere fluorescence thereby making it possible to provide a measure that is independent of slide-to-slide variations.

2˙3. *Spheroids.* – The human tumour cell line MG-63 will be used to grow spheroid aggregates. The levels of cell death induced by SOBP irradiation will be compared to those already obtained after 5 Gy of γ-rays. Cell death will be assessed by Hoechst 33258 staining. In the case of low LET irradiation, the expression of adhesion molecules was analysed using the inhibitor zVAD-fmk.

3. – Results

3˙1. *Transformation induction.* – CGL1 cells were irradiated at the medical exposure set-up at SIS, GSI. An extended volume simulating a tumour in a depth between 6 and 10 cm was irradiated with physical isodoses of 0.75 Gy. Survival and neoplastic transformation have been determined in six different positions of the beam ionisation profile

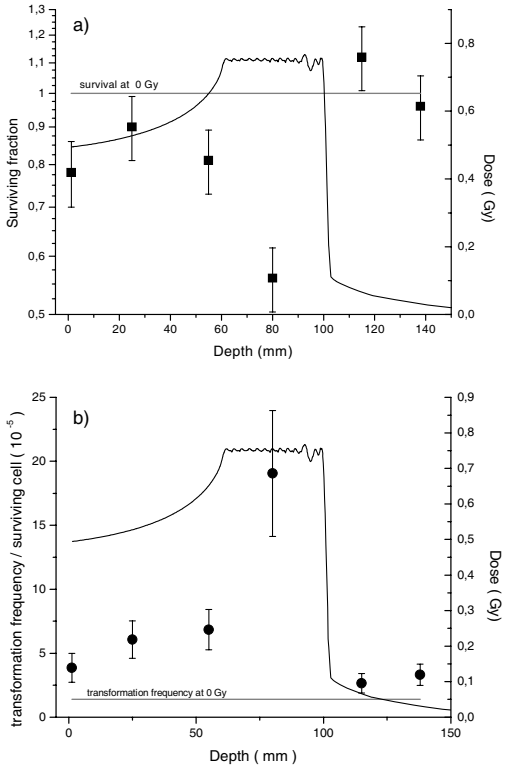

Fig. 1. – Survival (a) and neoplastic transformation (b) *vs.* depth along the ionisation path of the ^{12}C ion beam. The corresponding physical dose values are also reported (right axis).

from the entrance channel to the region behind the SOBP to evaluate the role of nuclear fragments. Figures 1a and 1b show survival and neoplastic transformation frequency at the various positions of the dose-depth curve. The corresponding physical dose values are also reported (right axis). Survival decreases with increasing depth reaching a minimum in the simulated tumour volume. Correspondingly, neoplastic transformation frequency increases with increasing depth reaching a maximum in same position. The decrease/increase in survival/transformation with depth is expected on the basis of the increase of dose and LET with increasing depth. These results are in line with those obtained in previous experiments on monoenergetic ^{12}C-ion beams with energy between 270 and 11.4 MeV/n (LET values between 13.8 and 172 keV/μm) [10]. In fact, it was found that the 270 MeV/n ^{12}C ions at the entrance channel show an oncogenic and killing potential very similar to that of photons, whereas the lowest energy /highest LET shows at low doses a RBE of about 7 and 12 for survival and transformation, respectively. Behind the tumour volume, where the physical dose is low compared with the entrance (a factor of about ten) and is due to nuclear fragments, survival is similar to the control values, *i.e.* 1; on the other end, transformation frequencies are greater than those relative to the controls and similar to the values at the entrance.

3˙2. *Radiation-induced premature senescence.* – Data from β-galactosidase assay clearly show that irradiation causes premature senescence (fig. 2). A greater proportion

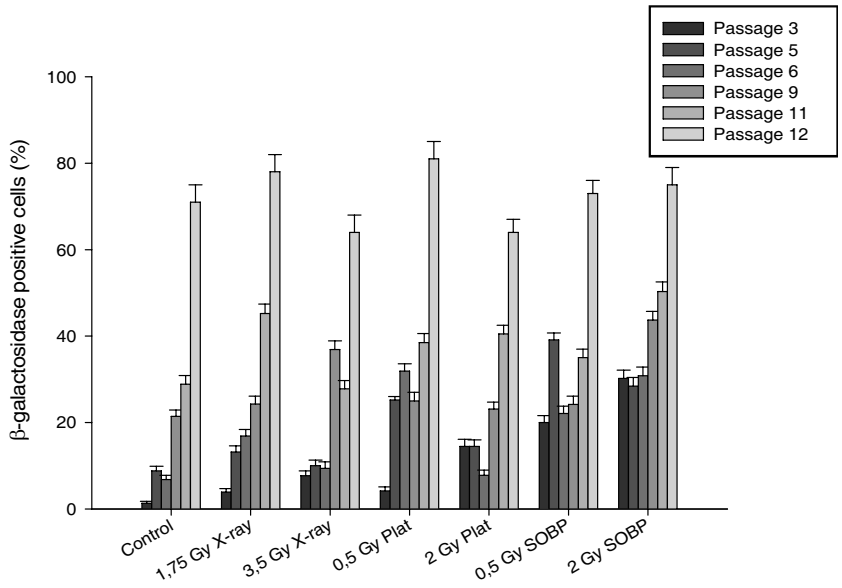

Fig. 2. – Fraction of senescent cells as a function of radiation quality and dose at various passages.

of cells expressing β-galactosidase activity was found amongst irradiated cells, exposed to both ^{12}C and X-ray, compared to the irradiated control from the same passage. The dependence upon dose or radiation quality is rather complex but points to a greater effectiveness of low dose *vs.* high dose in the earlier passages post-irradiation; at these passages and for the low doses used, high LET seems also to be more effective than low LET. At later times no clear dependence upon LET or dose was seen although descendants from irradiated cultures continued to exhibit a higher incidence of senescence than those from unirradiated controls. These data thus point to two temporally distinct waves of cellular senescence, an early radiation-induced senescence that is both LET and dose dependent, and a late senescence. These results are in qualitative agreement with data from Fournier *et al.* [34] on fibroblasts irradiated with various radiation qualities and are consistent with high doses of high LET radiation preferably inducing lethal effects while sub-lethal damage being able to induce premature senescence.

As regards the ratio T/C as an indicator of telomere length, a decrease was seen in ageing controls, thereby validating its use as a cytogenetic marker of senescence. Preliminary results indicate that a correlation between telomere shortening and radiation-induced premature senescence occurred for ^{12}C ion irradiation at the plateau position and for X-ray irradiation, although in the latter case an initial whereas no clear trend was observed for SOBP irradiation (fig. 3).

3˙3. *Three-dimensional approach.* – Previous studies with gamma-rays have shown that radiation-induced cell death levels depend on the three-dimensional organization of the irradiated cultures. When monolayers were irradiated, mitotic catastrophe was prevalent as mode of death, whereas apoptosis prevailed in the case of irradiated spheroids [35]. Protein analysis seems to corroborate the view that hypoxia may be involved in such processes.

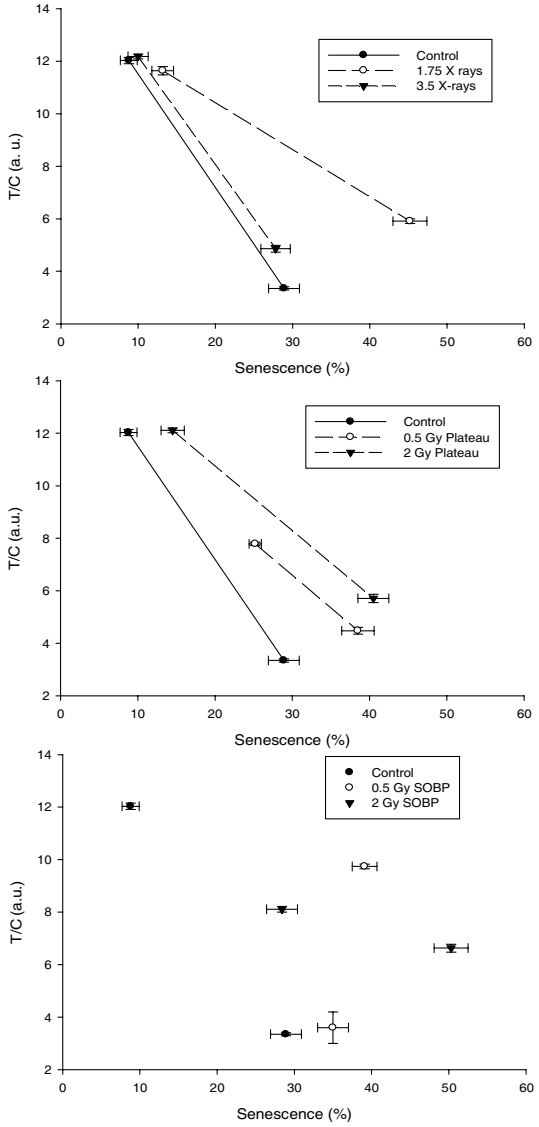

Fig. 3. – Relationship between telomere length, as measured by telomere to centromere fluorescence intensity ratio (T/C), and cellular senescence as assayed by β-galactosidase expression.

4. – Conclusions

The INFN-funded project ETIOPE aims at studying the effectiveness in the induction of neoplastic transformation and the onset of premature senescence in human cell lines by a therapeutic ^{12}C ion beam relative to photon irradiation.

Late-arising biologically relevant effects may be tumourigenic, *i.e.* leading to secondary cancers, possibly promoted by DNA lesions such as chromosome aberrations or non-targeted damage in the form of genomic instability, and non-tumourigenic. The latter can be brought forward by cell death or stress-induced cellular damage such as premature senescence.

As for neoplastic transformation, the results of the present study show that the oncogenic potential of ^{12}C ions in the SOBP is greater than at the beam entrance and that, as opposed to cell killing, the oncogenic effect of the low-dose nuclear fragments behind the tumour volume is not negligible. We are aware that these results pertain only to the probability of neoplastic transformation of a single surviving cell and many other aspects need to be taken into account in a therapy plan, nevertheless, as far as late effects are concerned, they suggest the necessity of restricting high LET irradiation to the tumour volume only and that the use of multiple opposing beam irradiation should be avoided whenever possible.

Our data demonstrate that ionising radiation is capable of causing normal human endothelial cells (HUVECs) to express markers of ageing earlier than do controls not exposed to radiation. Such a response, which follows a complex dependence upon radiation dose and quality, seems to occur in two phases: an early senescence appears to be more effectively induced by ^{12}C ions compared to X-rays as opposed to a delayed onset of LET-independent senescence. For a given LET, low doses are also more effective at causing such early senescence. From a mechanistic point of view, our data seem to support that radiation-induced premature senescence, at least in its early occurrence, is enacted via telomere shortening for ^{12}C ion irradiation in the plateau region, but do not suffice to ascertain whether a similar mechanism is in place in the case of SOBP irradiation. Altogether, these data seem to indicate that healthy tissues exposed to low doses of ^{12}C ions in the region of the dose-depth profile corresponding to the entrance beam channel, of interest for late effects of hadrontherapy patients, may be susceptible to premature senescence. Further experiments are warranted to confirm these results for the plateau region, to clarify the dependence upon telomere shortening of such a response in the case of SOBP and to examine the role of fragmentation in the onset of senescence.

The differential induction of cell death depending on the architecture of the irradiated cells shown in the case of low LET irradiation suggests testing the spheroid system with ^{12}C ions using isodoses in both the plateau and SOBP regions.

REFERENCES

[1] Amaldi U. and Kraft G., *J. Radiat. Res.*, **48** Suppl. A (2007) 27.
[2] Schulz-Ertner D., Jäkel O. and Schlegel W., *Semin. Radiat. Oncol.*, **16** (2006) 249.
[3] Tsujii H., Mizoe J., Kamada T., Baba M., Tsuji H., Kato H., Kato S., Yamada S., Yasuda S., Ohno T. *et al.*, *J. Radiat. Res.*, **48** Suppl. A (2007) 1.
[4] Durante M., *Radiother Oncol.*, **73** Suppl. 2 (2004) 158.
[5] Haettner E., Iwase H. and Schardt D., *Radiat. Prot. Dosimetry*, **122** (2006) 485.
[6] Hall E. J., *Int. J. Radiat. Oncol. Biol. Phys.*, **65** (2006) 1.
[7] Miller R. C., Marino S. A., Napoli J., Shah H., Hall E. J., Geard C. R. and Brenner D. J. *et al.*, *Int. J. Radiat. Biol.*, **76** (2000) 327.
[8] Stanbridge E. J., Der C. J., Doersen C. J., Nishimi R. Y., Peehl D. M., Weinstein B. E. and Wilkinson J. E., *Science*, **215** (1982) 252.
[9] Redpath J. L., Sun C., Colman M. and Stanbridge E. J., *Radiat. Res.*, **110** (1987) 468.
[10] Bettega D., Calzolari P., Hessel P., Stucchi C. and Weyrather W. K., to be published in *Int. J. Radiat. Oncol. Biol. Phys.*
[11] Bettega D., Calzolari P., Hessel P. and Weyrather W. K., GSI Scientific Report 2006 (2007-1) 350.
[12] Bettega D., Berlusconi C., Calzolari P., Hessel P. and Weyrather W. K., GSI Scientific Report 2007 (2008-1) 367.

[13] Blakeley E. A. and Kroneneberg A., *Radiat. Res.*, **150** (1998) S126.
[14] Yamada M., Wong F. L., Fujiwara S., Akahoshi M. and Suzuki G., *Radiat. Res.*, **161** (2004) 622.
[15] Jones K. R., Elmore L. W. and Jackson-Cook C., *Int. J. Radiat. Biol.*, **81** (2005) 445.
[16] Suzuki M., Tsuruoka C., Uchihori Y., Ebisawa S., Yasuda H. and Fujitaka K., *Radiat. Res.*, **164** (2005) 505.
[17] Campisi J., *Cell*, **120** (2005) 513.
[18] Weinberg R. A., *Nature*, **396** (1998) 23.
[19] Toussaint O., Medrano E. E. and Von Zglinicki T., *Exp. Gerontol.*, **35** (2000) 927.
[20] Suzuki K., Mori I., Nakayama Y., Miyakoda M., Kodama S. and Watanabe M., *Radiat. Res.*, **155** (2001) 248.
[21] Suzuki M. and Boothman D. A., *J. Radiat. Res.*, **49** (2008) 105.
[22] Collado M. and Serrano M., *Nature*, **6** (2006) 472.
[23] Shawi M. and Autexier C., *Mech. Ageing Dev.*, **129** (2008) 3.
[24] Baker D. G. and Krochak R. J., *Cancer Invest.*, **7** (1989) 287.
[25] Rhim J. S., Tsai W. P., Chen Z. Q., Chen Z., Van Waes C., Burger A. M. and Lautenberger J. A., *Carcinogenesis*, **19** (1998) 673.
[26] Hastings R., Qureshi M., Verma R., Lacy P. S. and Williams B., *Cell Prolif.*, **37** (2004) 317.
[27] Manti L., Durante M., Elsässer T., Gialanella G., Pugliese M., Ritter S., Scampoli P. and Grossi G., GSI Scientific Report 2006 (2007-1) 349.
[28] Dimri G. P., Lee X., Basile G., Acosta M., Scott G., Roskelley C., Medrano E. E., Linskensi M., Rubelj I., Pereira-Smith O., Peacocke M. and Campisi J., *Proc. Natl. Acad. Sci. USA*, **92** (1995) 9363.
[29] Narath R., Lörch T., Greulich-Bode K. M., Boukamp P. and Ambros P. F., *Cytometry A*, **68** (2005) 113.
[30] Narath R., Lörch T., Rudas M. and Ambros P. F., *Cytometry B*, **57** (2004) 15.
[31] Staab A., Zukowski D., Walenta S., Scholz M. and Mueller-Klieser W., *Radiat. Res.*, **161** (2004) 219.
[32] Mendonca M. S., Antoniono R. J., Sun C. and Redpath J. L., *Radiat. Res.*, **131** (1992) 345.
[33] Bettega D., Calzolari P., Piazzolla A., Tallone L. and Redpath J. L., *Int. J. Radiat. Biol.*, **72** (1997) 523.
[34] Fournier C., Winter M., Zahnreich S., Nasonova E., Melnikova L. and Ritter S., *Radiother. Oncol.*, **83** (2007) 277.
[35] Indovina P., Rainaldi G. and Santini M. T., *Int. J. Oncol.*, **31** (2007) 1473.